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(54) Astaxanthin Synthetase

(57) The present invention is directed to genetic materials useful for the preparation of astaxanthin from beta-carotene, such as polypeptides having astaxanthin synthase activity, DNA fragments coding for astaxanthin synthase, recombinant organisms and the like. Those novel genetic materials may be originated from Phaffia rhodozyma. The present invention also provides a process for the production of astaxanthin.

Description

[0001] The present invention relates to recombinant production of carotenoids and biological materials useful therefor.

[0002] Phaffia rhodozyma (P. rhodozyma) is a carotenogenic yeast strain which produces astaxanthin. Astaxanthin is distributed in a wide variety of organisms such as animal (birds such as flamingo and scarlet ibis, and fish such as rainbow trout and salmon), algae and microorganisms. It is also recognized that astaxanthin has a strong antioxidation property against oxygen radical, which is expected to apply for pharmaceutical usage to protect living cells against some diseases such as a cancer. Moreover, industrial need for astaxanthin as a coloring reagent is increasing, especially in the industry of farmed fish like salmon, because astaxanthin imparts distinctive orange-red coloration to the animals and contributes to consumer appeal in the marketplace.

[0003] P. rhodozyma is known as a carotenogenic yeast strain which produces astaxanthin. Different from the other carotenogenic yeast, Rhodotorula species, P. rhodozyma can ferment some sugars such as D-glucose. This is an important feature from a viewpoint of industrial application. In a recent taxonomic study, a sexual cycle of P. rhodozyma was revealed and its telemorphic state was designated under the name of Xanthophyllomyces dendrorhous (W. I. Golubev; Yeast 11, 101 - 110, 1995). Some strain improvement studies to obtain hyper producers of astaxanthin from P. rhodozyma have been conducted, but such efforts have been restricted to employ the method of conventional mutagenesis and protoplast fusion in this decade. Recently, Wery et al. developed a host vector system using P. rhodozyma in which a non-replicable plasmid was used to be integrated in multicopies into the genome of the ribosomal DNA of P. rhodozyma (Wery et al., Gene, 184, 89-97, 1997). Verdoes et al. reported more improved vectors to obtain a transformant of P. rhodozyma as well as its three carotenogenic genes which code for the enzymes that catalyzes the reactions from geranylgeranyl pyrophosphate to beta-carotene (WO 97/23633).

[0004] A specific biosynthetic pathway for carotenogenesis branches from general isoprenoid pathway at the point of important intermediate, farnesyl pyrophosphate (FPP) (FIG.1). FPP and IPP are condensed by geranylgeranyl pyrophosphate (GGPP) synthase which is coded by crtE in P. rhodozyma to produce GGPP. GGPP is then converted to beta-carotene by the sequential reaction of an enzyme functioning doubly as phytoene synthase and lycopene cyclase which is coded by crtBY and phytoene desaturase coded by crtI.

[0005] In bacteria, enzymes and genes which are involved in xanthophyll formation have been isolated and characterized in detail. beta-Carotene hydroxylase which is coded by crtZ is involved in the two steps of hydroxylation for beta-ionone-ring of beta-carotene at both of the ends. The crtZ gene was cloned from wide variety of organisms such as Erwinia uredovora (Misawa et al., J. Bacteriol., 172, 6704-6712, 1990), Flavobactor species (L. Pasamontes et al., 185 (1), 35-41, 1997) and Agrobacterium aurantiacum (Misawa et al., J. Bacteriol., 177 (22), 6575-6584, 1995). beta-Carotene ketolase which is coded by crtW catalyzes the two steps of introduction of oxo-group into beta-ionone -ring of beta-carotene at both of the ends. Kajiwara et al. cloned and sequenced bkt gene corresponding to crtW in eubacteria from Haematococcus bluvialis (Kajiwara et al., P. Mol. Biol., 29, 343-352, 1995). Harker et al. also cloned and sequenced crtO gene corresponding to crtW in eubacteria from Synechococcus PCC7942 (Harker et al., FEBS Letters, 404, 129-134, 1997). Both enzymes the hydroxylase and the ketolase have wide substrate specificity and this ensures the formation of wide variety of xanthophylls in case that both of the enzymes react at the same time depending on the reaction condition. (FIG.1)

[0006] As described above, all the genes which were involved in the formation of beta-carotene from FPP were isolated but the enzymes and genes which would be involved in the last step of xanthophyll formation from beta-carotene have not been identified on the protein and DNA level in P. rhodozyma. Although Johnson et al. (Crit. Rev. Biotechnol, 11 (4), 297 - 326, 191) proposed the existence of two independent pathways for astaxanthin formation by assuming that some of the xanthophyll compounds isolated by them would be intermediates of astaxanthin biosynthesis, such two independent pathways could not be proven because enzymes and genes which are involved in such pathways could not be isolated. Furthermore, it can not be excluded that these xanthophyll compounds could have been resulted from an experimental artifact in the isolation step of these compounds. Failure to isolate a mutant from P. rhodozyma which accumulates intermediates on the biosynthetic pathway from beta-carotene to astaxanthin made it difficult to clarify the biosynthetic pathway from beta-carotene to astaxanthin.

[0007] This invention relates to a gene and enzyme which is involved in the last step of the astaxanthin biosynthesis from beta-carotene to astaxanthin.

[0008] The present invention, provides an isolated DNA, specifically cDNA comprising a nucleotide sequence coding for astaxanthin synthase which is involved in the reaction from beta-carotene to astaxanthin in P. rhodozyma, like the AST gene.

[0009] In a preferred embodiment, the cloned DNA fragment can be characterized in that

(a) the said nucleotide sequence codes for the said enzyme having an amino acid sequence described in SEQ ID NO: 1, or

(b) the said nucleotide sequence codes for a variant of the said enzyme selected from (i) an allelic variant or (ii) an enzyme having one or more amino acid addition, insertion, deletion and/or substitution and having the stated enzyme activity.

[0010] In another preferred embodiment, the isolated cDNA fragment can be derived from a gene of Phaffia rhodozyma and is selected from:

(i) a cDNA sequence represented by SEQ ID NO: 2;
(ii) an isocoding or an allelic variant for the cDNA sequence represented by SEQ ID NO: 2; and
(iii) a derivative of a cDNA sequence represented by SEQ ID NO: 2 with addition, insertion, deletion and/or substitution of one or more nucleotide(s), and coding for a polypeptide having the said enzyme activity.

[0011] In another preferred embodiment the present invention is directed to the isolated cDNA as described above, which is characterized in that the said nucleotide sequence is:

(i) a nucleotide sequence represented in SEQ ID NO: 2;
(ii) a nucleotide sequence which, because of the degeneracy of the genetic code, encodes an astaxanthin synthase having the same amino acid sequence as that encoded by the nucleotide sequence (i); and
(iii) a nucleotide sequence which hybridizes to the complement of the nucleotide sequence from i) or ii) under standard hybridizing conditions.

[0012] In still another preferred embodiment, the isolated genomic DNA fragment can be derived from a gene of Phaffia rhodozyma and is selected from:

(i) a genomic DNA sequence represented by SEQ ID NO: 3;
(ii) an isocoding or an allelic variant for the genomic DNA sequence represented by SEQ ID NO: 3; and
(iii) a derivative of a genomic DNA sequence represented by SEQ ID NO: 3 with addition, insertion, deletion and/or substitution of one or more nucleotide(s), and coding for a polypeptide having the said enzyme activity.

[0013] In another preferred embodiment the present invention is directed to the isolated genomic DNA as described above, which is characterized in that the said nucleotide sequence is:

(i) a nucleotide sequence represented in SEQ ID NO: 3;
(ii) a nucleotide sequence which, because of the degeneracy of the genetic code, encodes an astaxanthin synthase having the same amino acid sequence as that encoded by the nucleotide sequence (i); and
(iii) a nucleotide sequence which hybridizes to the complement of the nucleotide sequence from i) or ii) under standard hybridizing conditions.

[0014] Another aspect of the present invention, provides a recombinant polypeptide having an astaxanthin synthase activity involved in the reaction from beta-carotene to astaxanthin in P. rhodozyma which is obtainable by the expression of the cloned DNA fragment as set forth above.
[0015] A preferred embodiment of the recombinant polypeptide of the present invention is characterized in that

(a) the said polypeptide has an amino acid sequence as described in SEQ ID NO: 1, or
(b) the said polypeptide is a variant of the peptide defined in (a) which is selected from (i) an allelic variant or (ii) an enzyme having one or more amino acid addition, insertion, deletion and/or substitution and having the stated enzyme activity.

[0016] Therefore the present invention is also directed to variants of the polypeptides of the present case. Such variants are defined on the basis of the amino acid sequence of the present invention by addition, insertion, deletion and/or substitution of one or more amino acid residues of such sequences wherein such derivatives still have the same type of enzymatic activity as the corresponding polypeptides of the present invention or they are the result of the well known phenomenon of allelic variation. Such activities can be measured by any assays known in the art or specifically described herein. Such variants can be made either by chemical peptide synthesis known in the art or by recombinant means on the basis of the DNA sequences as disclosed herein by methods known in the state of the art, such as, e.g. that disclosed by Sambrook et al. (Molecular Cloning, Cold Spring Harbour Laboratory Press, New York, USA, second edition 1989). Amino acid exchanges in proteins and peptides which do not generally alter the activity of such molecules are known in the state of the art and are described, for example, by H. Neurath and R.L. Hill in "The Proteins"

(Academix Press, New York, 1979, see especially Figure 6, page 14). The most commonly occurring exchanges are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/thr, Ser/Asn, Ala/Val, Ser/Gly, Thy/Phe, Ala/Pro, Lys/Art. Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly as well as the reverse.

[0017] Furthermore the present invention is not only directed to the DNA sequences as disclosed e.g., in the sequence listing as well as their complementary strands, but also to those which include these sequences, DNA sequences which hybridize under Standard Conditions with such sequences or fragments thereof and DNA sequences, which because of the degeneration of the genetic code, do not hybridize under Standard Conditions with such sequences but which code for polypeptides having exactly the same amino acid sequence.

[0018] "Standard Conditions" for hybridization mean in this context the conditions which are generally used by a man skilled in the art to detect specific hybridization signals and which are described, e.g. by Sambrook et al., (s.a.) or preferably so-called stringent hybridization and non-stringent washing conditions, or more preferably so-called stringent hybridization and stringent washing conditions a man skilled in the art is familiar with and which are described, e.g., in Sambrook et al. (s.a) or more preferably so-called medium stringent conditions, e.g. using the DIG (digoxigenin) Labeling kit and luminescent detection kit of Boehringer Mannheim (Mannheim, Germany) following the protocol given by the manufacturer and using as the hybridization solution:

formamide (WAKO, Osaka, Japan) 50% (V/V)
5 x SSC
blocking reagent (Boehringer) 2% (W/V)
N-lauroylsarcosine 0.1% (W/V)
SDS 0.3% (W/V)

at a temperature of 42°C over night and subsequently washing and detection as indicated by the manufacturer.

[0019] DNA sequences which are derived from the DNA sequences of the present invention either because they hybridize with such DNA sequences (see above) or can be constructed by the polymerase chain reaction by using primers designed on the basis of such DNA sequences can be prepared either as indicated namely by the PCR reaction, or by site directed mutagenesis [see e.g., Smith, Ann. Rev. Genet. 19, 423 (1985)] or synthetically as described, e.g., in EP 747 483 or by the usual methods of Molecular Cloning as described, e.g., in Sambrook et al. (s.a.).

[0020] The present invention is also directed to a vector or plasmid comprising a DNA as described above and a host cell transformed or transfected by a DNA as described above or a vector or plasmid as indicated above.

[0021] The present invention also provides a recombinant organism which is obtainable by the transformation of a host using a recombinant DNA carrying the DNA as mentioned above.

[0022] The present invention also relates to a method for producing an enzymatic polypeptide capable of catalyzing the reaction from beta-carotene to astaxanthin, which comprises culturing a recombinant organism described above under the condition conductive to the production of said enzymatic polypeptide.

[0023] In a further aspect the present invention provides a method for the production of astaxanthin which comprises introducing one or more of the DNA described above into an appropriate host organism and cultivating this transformed organism under the conditions conductive to the production of astaxanthin.

[0024] The enzymatic polypeptide of the present invention will be also useful for a method for the production of astaxanthin, which method comprises contacting beta-carotene with the recombinant polypeptide having an astaxanthin synthase activity involved in the reaction from beta-carotene to astaxanthin as set forth above in the presence of an appropriate electron donor in an appropriate reaction mixture containing an appropriate reconstituted membrane. In this method, said recombinant polypeptide may be present in the form of a reconstituted membrane which is prepared from biological membranes like microsomes or mitochondrial membranes. The recombinant polypeptide may be also present in the form of a reconstituted artificial membrane, like liposomes. The electron donor like cytochrome P450 reductase is an appropriate electron donor which can reduce a reaction center of the enzyme of the present invention.

[0025] The following drawings are included to further illustrate the present invention together with the detailed description given below.

FIG. 1 shows the biosynthetic pathway from acetyl-CoA to astaxanthin in P. rhodozyma.
FIG. 2 shows a restriction map of the plasmid pR16 which harbors a partial genomic AST gene.

[0026] FIG. 3 shows the expression study for AST gene to which 6 x His were added at its amino terminal end on removal of its transmembrane domain. The cells from 0.1 ml of broth were subjected to 10 % sodium dodesyl sulfide - polyacrylamide gel electrophoresis (SDS-PAGE). Lane 1, Molecular weight marker (105 kDa, 82.0 kDa, 49.0 kDa and 33.3 kDa, up to down, Bio-RAD, Richmond, U.S.A.); Lane 2, E. coli (BL21 (DE3) (pLysS) (pAST315) without IPTG); Lane 3, E. coli (BL21 (DE3) (pLysS) (pAST315) with 1.5 mM IPTG); Lane 4, Molecular weight marker).

[0027] In general, there are a number of methods to clone a gene coding for biosynthetic enzymes. For example,

degenerate PCR can be used. Degenerate PCR is a method to clone a gene of interest which has high homology of its encoded amino acid sequence to the one of a known enzyme from other species which has the same or similar function. A degenerate primer, which is used as a set of primers in degenerate PCR, was designed by a reverse translation of the amino acid sequence to corresponding nucleotides ("degenerated"). In such a degenerate primer, a mixed primer which consists any of A, C, G or T, or a primer containing inosine at an ambiguity code is generally used. After cloning of a partial fragment of the gene of interest the genetic fragment containing the entire gene can be screened by using the cloned and labeled partial DNA fragment as a probe.

[0028] In the case of cloning a gene encoding an enzyme whose activity can be measured by an enzymatic assay, purification of such an enzyme by monitoring enzyme activity and determination of its amino acid sequence for the enzyme is a good method. An amino acid sequence thus obtained is easily translated in reverse into the corresponding nucleotide sequence(s). A DNA fragment which has the corresponding nucleotide sequence can be synthesized in vitro with a DNA synthesizer and labeled for direct usage as a hybridization probe. An alternative way to obtain a hybridization probe is the degenerate PCR method using the amino acid sequence information.

[0029] To clone a gene whose function can not be characterized enzymatically, a method called shot-gun screening has been employed as a conventional cloning method. This method comprises the isolation of a mutant strain which lacks the specific gene coding for any of the biosynthetic enzymes of interest, [and the transformation of the mutant strain by the DNA prepared from the organism that has an intact gene which corresponds to the gene as such mutated. For an isolation of such a mutant, conventional mutagenesis is often used. Confirmation of the acquired phenotype which is the same as that of the parent strain can be performed by examination of its auxotrophy and the like. In the case that the donor DNA contains the gene which corresponds to the mutated gene in the mutant strain, the transformant by such a gene acquired the same phenotype as the parent strain as a result of genetic complementation.

[0030] As a vector, any form of vectors whether they can replicate or not in the cloning host, can be used for shot gun cloning. For the usage of a replicative vector, the capability of complementation is a requisite and it is not necessary that such a vector contains a homologous sequence to the genome of the recipient. In the case of using a non-replicative vector, it is necessary that such a vector contains a homologous sequence to the genome of the host to make a recombination between donor and recipient DNAs.

[0031] For the cloning of the DNAs of the present invention the method called "color complementation" can be employed. Non carotenogenic organisms, such as Escherichia coli can acquire the carotenogenic ability as a result of transformation by carotenogenic genes which could be cloned from carotenogenic organisms such as Erwinia uredovora, Erwinia herbicola and the like. E. coli harboring crtE, crtB, crtI and crtY can produce beta carotene and color the cells in yellow. Exploiting such a characteristics, numbers of carotenogenic genes have been cloned from various carotenogenic organisms such as bacteria and plants. For example, to clone the crtY gene coding for lycopene cyclase, E. coli harboring crtE, crtB and crtI on the compatible vector against pUC vector is prepared as a transformation host. Such a host turns red which shows accumulation of lycopene. Next, a cDNA or genomic library from carotenogenic organisms can be constructed using the pUC vector. If the gene corresponding to crtY in the donor carotenogenic organism is present in the transformed plasmid, genetic complementation would occur and E. coli would turn yellow which would show the acquisition of the ability to produce beta-carotene. In fact, crtE, crtBY and crtI gene were cloned from P. rhodozyma by this method (Verdoes et al., WO97/23633).

[0032] Regarding the cloning of the gene which is involved in the reaction from beta-carotene to astaxanthin, Kajiwara et al. constructed a cDNA expression library from P. rhodozyma in the host E. coli harboring crtE, crtB, crtI and crtY genes from E. uredovora (Kajiwara et al., WO 96/28545, 1996). In such a cloning system, a gene which is involved in the reaction from beta-carotene to astaxanthin could be theoretically cloned from P. rhodozyma by judging red pigmentation which shows the accumulation of canthaxanthin or astaxanthin. However, such a gene has not been reported so far. Many researchers speculate about the possibility that membrane-bound carotenogenic enzymes would form an enzyme complex. In such a model, the affinity among carotenogenic enzymes would be necessary for efficient carotenogenesis. Based on such an assumption, this color complementation method is not suitable to clone the enzyme involved in the last step of astaxanthin biosynthesis, namely the one from beta-carotene to astaxanthin, because exogeneous enzymes might not have affinity to the Phaffia's carotenogenic enzyme in the sequential reaction of the carotenogenesis.

[0033] In this invention, P. rhodozyma ATCC96815 which has been redeposited as a Budapest Treaty deposit at the American Type Culture Colleciton (ATCC) under accession number 74486 on February 18, 1999 and which is blocked for the reaction from beta-carotene to astaxanthin was used as a transformation host (Schroeder, W.A. and Johnson, E.A., J. Ind. Microbiol. 14 502-507, 1995). Transformation of this mutant by the genomic library prepared from the chromosome of a wild type strain of P. rhodozyma ATCC96594 which has also been redeposited as a Budapest Treaty deposit at the American Type Culture Collection (ATCC) under accession number 74438 on April 8, 1998 was used to isolate a clone which produces astaxanthin. In the present invention, such genetic fragment complementing the reaction from beta-carotene to astaxanthin in P. rhodozyma was isolated and its nucleotide sequence was determined.

[0034] Such a gene / DNA of the present invention can be used for overproduction of astaxanthin through a gene

dosage effect using gene amplification or promoter modification other than complementation of blocked mutation.

[0035] In general, a gene consists of several parts which have different functions. In eukaryotes, genes which encode corresponding protein are transcribed to premature messenger RNA (pre-mRNA), differing from the genes for ribosomal RNA (rRNA), small nuclear RNA (snRNA) and transfer RNA (tRNA). Although RNA polymerase II (PolII) plays a central role in this transcription event, PolII cannot solely start a transcription without a cis element covering an upstream region containing a promoter and an upstream activation sequence (UAS), and a trans-acting protein factor. At first, a transcription initiation complex which consists of several basic protein components recognize the promoter sequence in the 5'-adjacent region of the gene to be expressed. In this event, some additional participants are required in the case of the gene which is expressed under some specific regulation, such as a heat shock response, or adaptation to a nutrition starvation, and so on. In such a case, a UAS is required to exist in the 5'-untranslated upstream region around the promoter sequence, and some positive or negative regulator proteins recognize and bind to the UAS. The strength of the binding of transcription initiation complex to the promoter sequence is affected by such a binding of the trans-acting factor around the promoter, and this enables the regulation of the transcription activity.

[0036] After the activation of a transcription initiation complex by the phosphorylation, a transcription initiation complex initiates a transcription from the transcription start site. Some parts of the transcription initiation complex are detached as an elongation complex from the promoter region to the 3' direction of the gene (this step is called as a promoter clearance event) and an elongation complex continues the transcription until it reaches to a termination sequence that is located in the 3'-adjacent downstream region of the gene. Pre-mRNA thus generated is modified in nucleus by the addition of cap structure at the cap site which almost corresponds to the transcription start site, and by the addition of polyA stretches at the polyA signal which locates at the 3'-adjacent downstream region. Next, intron structures are removed from coding region and exon parts are combined to yield an open reading frame whose sequence corresponds to the primary amino acid sequence of a corresponding protein. This modification in which a mature mRNA is generated is necessary for a stable gene expression. cDNA in general terms corresponds to the DNA sequence which is reverse-transcribed from this mature mRNA sequence. It can be synthesized by the reverse transcriptase derived from viral species by using a mature mRNA as a template, experimentally.

[0037] In this invention, mutation point of the P. rhodozyma ATCC96815 strain which rendered beta-carotene production to P. rhodozyma wild type strain was determined. From the sequencing result, it was suggested that the base change at the splicing sequence of the eighth intron of AST gene cause such a phenotype as specific beta-carotene accumulation through the improper splicing of mRNA. RT-PCR analysis detected the improper spliced product for AST gene and strongly supported the identification of the mutation point.

[0038] This invention also provides the recombinant AST gene which can be expressed in different host organisms such as E. coli. In this invention, such recombinant AST gene was expressed in E. coli and it was confirmed that AST gene encoded protein product whose size was corresponded to the deduced molecular weight. Biological production of astaxanthin can be realized by using novel AST gene and such recombinant DNA techniques.

[0039] According to the present invention, the gene coding for the enzyme which is involved in the last step of astaxanthin biosynthesis was cloned from a cDNA library of P. rhodozyma, and their nucleotide sequence was determined. Furthermore, a part of genomic DNA including promoter and terminator was cloned and can be used for a cloning of its entire gene including the promoter and terminator region.

[0040] An entire gene with its coding region, its intron as well as its regulation regions such as a promoter and terminator can be cloned by screening a genomic library, which has been constructed in a phage or plasmid vector in an appropriate host by using a labeled cDNA fragment as a screening probe. Generally, one of the most common host strains for the construction of a genomic library is E. coli. As a vector a phage vector, such as a lambda phage vector, or a plasmid vector such as a pUC vector can be used. A genomic library constructed in this way, e.g. from P. rhodozyma DNA can be screened by using a labeled DNA fragment with a portion of the gene of interest as a probe. Hybridized plaques or colonies can then be picked and used for subcloning and/or determination of the nucleotide sequence.

[0041] There are several strategies to enhance the desired enzymatic activity of the protein of interest by using its DNA sequence.

[0042] One strategy is to use the gene itself in its native form. The simplest approach is to amplify the genomic sequence including its regulatory sequences such as the promoter and the terminator. This can be done by cloning of the genomic fragment coding for the enzyme of interest into an appropriate vector with a selectable marker which functions in P. rhodozyma. A drug resistance gene coding for an enzyme that enables the host to survive in the presence of a toxic antibiotic is often used as a selectable marker. The G418 resistance gene harbored in pGB-Ph9 (Wery et al., Gene, 184, 89-97, 1997) is an example of such a vector construction. As a vector, two types of vectors can be commonly used. One of these types is an integration vector which does not have an autonomous replicating sequence. The plasmid pGB-Ph9 is an example of this type of vectors. Because such a vector does not have an autonomous replicating sequence, the above vector cannot replicate by itself and can be present only in an integrated form on the chromosome of the host as a result of a single-crossing recombination using the homologous sequence between a vector and

the chromosome. By increasing the concentration of the corresponding drug in the selection medium, the strain in which the integrated gene is amplified on the chromosome can only survive. Another type of vector is a replicable vector which has an autonomous replicating sequence. Such a vector can exist in a multicopy state. In this type of vector, a nutrition complementation maker can be also used in the host which has an appropriate auxotrophy marker. The P. rhodozyma ATCC24221 strain which requires cytidine for its growth is one example of such an auxotroph. By using a CTP synthetase as a donor DNA for ATCC24221, a host vector system using a nutrition complementation can be established.

[0043] Another strategy to overexpress an enzyme of interest is the placement of the gene of interest under a strong promoter. In such a strategy, the gene of interest must not necessarily be in a multicopy state. Furthermore, a promoter whose promoter activity is induced in an appropriate growth phase and an appropriate timing of cultivation can be also used. Production of astaxanthin accelerates in the late phase of the growth, such as the production phase of a secondary metabolite. For example, by placing carotenogenic genes under the control of a vegetative promoter, the gene expression of these genes could be induced in the exponential growth phase and the production of astaxanthin can become associated to the growth of the production strain.

[0044] In this invention, promoter and terminator fragment for triose phosphate isomerase (TPI) gene was cloned from P. rhodozyma as one example of such constitutive promoter and terminator. Moreover, a restoration of astaxanthin production was confirmed in the transformants in which AST gene was expressed on the different locus (AMY locus which lies amylase gene) on the chromosome of beta-carotene producing P. rhodozyma ATCC96815 driven by constitutive promoter and terminator derived from TPI gene.

[0045] Still another strategy to overexpress enzymes of interest is mutation in its regulatory elements. For this purpose, a kind of reporter gene, such as the beta-galactosidase gene, luciferase gene, a gene coding for a green fluorescent protein, and the like is inserted between the promoter and the terminator sequence of the gene of interest so that all the parts including promoter, terminator and the reporter gene are fused and function together. A transformed P. rhodozyma in which said reporter gene is introduced on the chromosome or on the vector can be mutagenized in vivo to induce a mutation within the promoter region of the gene of interest. The mutation can be monitored by detecting the change of the activity coded for by the reporter gene. If the mutation occurs in a cis element of the gene, the mutation point would be determined by the rescue of the mutagenized gene and sequencing. This mutation can then be introduced to the promoter region on the chromosome by the recombination between the native and the mutated promotor sequence. In the same way a mutation in the gene which codes for a trans-acting factor can be made.

[0046] A mutation can be also induced by an in vitro mutagenesis of a cis element in the promoter region. In this approach, a gene cassette, containing a reporter gene which is fused to a promoter region derived from a gene of interest at its 5'-end and a terminator region from a gene of interest at its 3'-end, is mutagenized and then introduced into P. rhodozyma. By detecting the difference of the activity of the reporter gene, an effective mutation would be screened. Such a mutation can be introduced in the sequence of the native promoter region on the chromosome by the same method as the case of an in vivo mutation approach.

[0047] As donor DNA, a gene coding for an enzyme which catalyzes the reaction from beta-carotene to astaxanthin could be introduced. A coding sequence which is identical to its native sequence, as well as its allelic variant, a sequence which has one or more amino acid additions, deletions and/or substitutions as far as its corresponding enzyme has the same type of enzyme activity, can be used. Such a vector can then be introduced into P. rhodozyma by transformation and the transformants can be selected by spreading the transformed cells on an appropriate selection medium such as YPD agar medium containing geneticin in the case of pGB-Ph9 or a minimal agar medium omitting cytidine in the case of using auxotroph ATCC24221 as a recipient.

[0048] Such a genetically engineered P. rhodozyma can be cultivated in an appropriate medium and evaluated for its productivity of astaxanthin. A hyper producer of astaxanthin thus selected would be confirmed in view of the relationship between its productivity and the level of gene or protein expression which is introduced by such a genetic engineering method.

<u>Examples</u>

[0049] The following materials and methods were employed in the specific Examples described below:

<u>Strains</u>:

[0050]

P. rhodozyma ATCC96594 (This strain has been redeposited on April 8, 1998 as a Budapest Treaty deposit under accession No. 74438)

P. rhodozyma ATCC96815 (This strain has been redeposited on February 18, 1999 as a Budapest Treaty deposit under accession No. 74486)

E. coli DH5alpha F, phi80d, lacZdeltaM15, delta(lacZYA-argF)U169, hsd (r_K^-, m_K^+), recA1, endA1, deoR, thi-1, supE44, gyrA96, relA1 (Toyobo, Osaka, Japan)

E. coli XL1-Blue MRF': delta(mcrA)183, delta(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac[F' proAB, $lacI^qZ$deltaM15, Tn10 (tet^r)] (Stratagene, La Jolla, USA)

E. coli SOLR: $e14^-$(mcrA), delta(mcrCB-hsdSMR-mrr)171, sbcC, recB, recJ, umuC :: Tn5(kan^r), uvrC, lac, gyrA96, relA1, thi-1, endA1, $lambda^R$, [F' proAB, $lacI^qZ$deltaM15] Su^-(nonsuppressing) (Stratagene)

E.coli TOP10: F^-, mcrA, delta(mrr-hsdRMS-mcrBC), phi80, delta(lacZ M15), delta(lacX74), recA1, deoR, araD139, (ara-leu)7697, galU, galK, rpsL(Str^r), endA1, nupG (Invitrogen, NV Leek, Netherlands)

E. coli BL21 (DE3) (pLysS): dcm^-, $ompTr_B^-m_B^-$, lon^- lambda(DE3), pLysS (Stratagene)

Vectors:

[0051]

pUC19 (Takara Shuzo, Otsu, Japan)
lambdaZAPII (Stratagene)
pCR2.1-TOPO (Invitrogen)
pET11c (Stratagene)

Media

[0052] P. rhodozyma strain is maintained routinely in YPD medium (DIFCO, Detroit, USA). E. coli strain is maintained in LB medium (10 g Bacto-trypton, 5 g yeast extract (DIFCO) and 5 g NaCl per liter). When an agar medium was prepared, 1.5 % of agar (WAKO, Osaka, Japan) was supplemented.

Methods

[0053] General molecular biology methods were done according to those described in Molecular cloning: a Laboratory Manual, 2nd Edition (Cold Spring Harbor Laboratory Press, 1989). Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo.

[0054] Isolation of chromosomal DNA from P. rhodozyma was performed by using QIAGEN Genomic Kit (QIAGEN, Hilden, Germany) following the protocol supplied by the manufacturer. Mini-prep of plasmid DNA from transformed E. coli was performed with the Automatic DNA isolation system (PI-50, Kurabo, Co. Ltd., Osaka, Japan). Midi-prep of plasmid DNA from an E. coli transformant was performed by using QIAGEN column (QIAGEN). A DNA fragment was isolated and purified from agarose by using QIAquick or QIAEX II (QIAGEN).

[0055] Fluorescent DNA primers for DNA sequencing were purchased from Pharmacia. DNA sequencing was performed with the automated fluorescent DNA sequencer (ALFred, Pharmacia, Uppsala, Sweden).

[0056] Competent cells of DH5alpha were purchased from Toyobo.

[0057] The apparatus and reagent for biolistic transformation of P. rhodozyma were purchased from Nippon Bio-Rad Laboratories (Tokyo, Japan).

Example 1

Isolation of genomic DNA from P. rhodozyma

[0058] To isolate a genomic DNA from P. rhodozyma, ATCC96594 the QIAGEN genomic kit was used according to the method specified by the manufacturer.

[0059] At first, cells of P. rhodozyma ATCC96594 from 100 ml of overnight culture in YPD medium were harvested by centrifugation (1500 x g for 10 min.) and washed once with TE buffer (10 mM Tris / HCl (pH 8.0) containing 1 mM EDTA). After suspending in 8 ml of Y1 buffer of the QIAGEN genomic kit, lyticase (SIGMA, St. Louis, USA) was added at the concentration of 2 mg/ml to disrupt cells by enzymatic degradation and the reaction mixture was incubated for 90

minutes at 30 °C and then proceeded to the next extraction step. Finally, 20 μg of genomic DNA was obtained.

Example 2

Construction of a genomic library from P. rhodozyma ATCC96594

[0060] As described in the section "detailed description of the invention", a plasmid harboring a drug resistant marker cassette was constructed by inserting a G418 resistant structure gene between the promoter and terminator region of the gene of the glyceraldehyde-3-phosphate dehydrogenase (GAP) and ligating this cassette into the KpnI- and HindIII-digested pUC19. This plasmid was named pUC-G418 and used further on. Then, a ClaI linker was ligated into the unique EcoRI site of the pUC-G418 vector and the resultant plasmid pUC-G418C1512 was obtained and used as a vector backbone in the construction of the Phaffia's genomic library.

[0061] Then, 10 μg of chromosomal DNA prepared from P. rhodozyma ATCC96594 as described above in Example 1 was digested partially with 1.6 units of HpaII for 45 minutes at 37 °C and was subjected to agarose gel electrophoresis. After staining by ethidium bromide, partially digested DNA species from 4 to 10 kb were recovered by electroelution using a dialysis membrane. After ethanol precipitation of recovered HpaII fragments, 1.215 μg of DNA was obtained.

[0062] Next, 3 μg of pG418C1512 was digested by 10 units of ClaI for an hour at 37 °C and precipitated with ethanol. ClaI-digested pG418C1512 was then dephosphorylated using calf intestine alkaline phosphatase. ClaI-digested and dephosphorylated pG418C1512 vector was then subjected to agarose gel electrophoresis and the DNA fragment was recovered using the QIAquick protocol according to the instructions of the manufacturer. Finally, 2.62 μg of ClaI-digested and dephosphorylated pG418C1512 was obtained.

[0063] 2.62 μg of ClaI-digested and dephosphorylated pG418C1512 was ligated to 1.22 μg of HpaII-partially digested Phaffia's genomic DNA over night at 16 °C and the resultant ligation solution was used as donor DNA for the transformation of an E. coli DH5alpha strain. The total ligation mixture (270 μl) was transferred to 1 ml of competent cells of DH5a (Toyobo). After a heat shock treatment at 42 °C for 45 seconds succeeding by maintenance on ice for 30 minutes, the transformed cells were placed on ice for 2 minutes and then incubated at 37 °C for an hour with the addition of 5 ml of SOC medium:

0.5% yeast extract (DIFCO)
2% trypton (DIFCO)
10mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
20 mM $MgSO_4$
20 mM glucose.

[0064] Thus incubated cells were transferred into 100 ml of LB medium containing 100 gamma/ml of ampicillin. Cultivation was continued overnight at 37 °C and then the cells were harvested for plasmid midi-preparation.

[0065] A plasmid library was prepared from harvested cells using QIAGEN midi-prep columns according to the method supplied by the manufacturer. Finally, 0.3 mg/ml of Phaffia's genomic library was obtained in the total volume of 5 ml and used as a genomic library in the further study.

Example 3

Transformation of P. rhodozyma ATCC96815 with a biolistic method

[0066] Transformation was done according to the method described in Methods in Molecular Biology (Johnston et al., 53; 147-153, 1996). As a host strain, P. rhodozyma ATCC96815 was cultured in YPD medium to the stationary phase. After centrifugation of the broth, cells were concentrated 10-fold with sterilized water and 200 μl of the cell suspension was spread on YPD medium containing 100 gamma/ml of geneticin, and 0.75 M of mannitol and sorbitol. Five micrograms of a genomic library, prepared as described in Example 2, was coated on 1.5 mg of 0.9 μm gold particle, and used as donor DNA for the biolistic transformation. Approximately twenty thousands of geneticin-resistant clones were yielded (300 to 500 colonies per plate) after one week of incubation at 20 °C. Although most of the transformants showed a yellow color (as the host strain, ATCC96815 did), three colonies pigmented red and were used further on.

Example 4

Analysis of carotenoid obtained from red-pigmented transformants

[0067] Red-pigmented transformants obtained from P. rhodozyma ATCC96815 were cultivated in 10 ml of YPD medium at 20 °C in test tubes. Then, cells were harvested from 0.5 ml of broth and used for the extraction of carotenoids from cells. The carotenoid content of P. rhodozyma was measured by HPLC after extraction of the carotenoids from cells of P. rhodozyma by disruption with glass beads as described. After extraction, disrupted cells were then collected by centrifugation and the resultant supernatant was analyzed for carotenoid content with HPLC.

HPLC column; Chrompack Lichrosorb si-60 (4.6 mm, 250 mm)

Temperature; room temperature

Eluent; acetone / hexane (18 / 82) add 1 ml / L of water to elluent

Injection volume; 10 μl

Flow Rate; 2.0 ml/minute

Detection; UV at 450 nm

[0068] A sample of beta-carotene was purchased from SIGMA and astaxanthin was obtained from Hoffman La-Roche (Basel, Switzerland).

[0069] As a result of HPLC analysis, it was confirmed that all three red transformants produced astaxanthin specifically though the host strain, ATCC96815 produced only beta-carotene.

Example 5

Plasmid rescue from the chromosome of red transformants which produced astaxanthin

[0070] Chromosomal DNA was prepared from all the astaxanthin-producing transformants. For this purpose, the QIAGEN genomic kit was used according to the method specified by the manufacturer, as described in Example 1. 5 μg of chromosomal DNA, thus prepared, was digested by HindIII and then purified according the QIAquick protocol. E. coli DH5alpha competent cells were transformed by the ligated DNA solutions and then spread on LB agar medium containing 100 μg/ml of ampicillin. All of the transformants had the same insert fragments in their plasmids, judging from sequence analysis of the plasmids. This indicated that three independent red transformants derived from P. rhodozyma ATCC96815 were yielded by the same type of recombination event between the donor DNA of the genomic library and chromosomal DNA. One of the plasmids thus rescued was named pR2-4 and used further on.

Example 6

Screening of the original genomic library by using pR2-4 as a hybridization probe

[0071] Because the rescued fragment in pR2-4 may have mutations depending on the direction of the recombination event yielding red transformants of P. rhodozyma, screening of the original genomic library was done by using pR2-4 as a hybridization probe.

[0072] For this purpose, twenty thousand E. coli transformants of the original genomic library, as described in Example 2 were transferred to nylon membrane filters (Hybond-N+, Amersham, Buckinghamshire, UK) and subjected to colony hybridization. Three transformants which harbored the same nucleotide sequence in their insert as that of pR2-4 were isolated. The isolated plasmids from these transformants were named pR3, pR5.1 and pR16.

[0073] Next, P. rhodozyma ATCC96815 was transformed by pR3, pR5.1 and pR16. All the transfomants colored red. This result suggests that the isolated plasmids might contain the gene encoding an enzyme involved in the reaction of beta-carotene to astaxanthin in P. rhodozyma. We designated this gene as AST gene. Among these plasmids, pR16 was used further on.

Example 7

Isolation of mRNA from P. rhodozyma for cDNA analysis

[0074] To analyze the pattern of transcripts from P. rhodozyma, total RNA was isolated from P. rhodozyma ATCC96594 and ATCC96815 by phenol extraction by combination of the cell disruption with glass beads and purified mRNA using an mRNA separation kit (Clontech, Palo Alto, USA).

[0075] At first, cells of ATCC96594 and ATCC96815 strains from 10 ml of a two-day-culture in YPD medium were harvested by centrifugation (1500 x g for 10 min.) and washed once with extraction buffer (100 mM Tris / HCl (pH 7.5) containing 0.1 M LiCl and 0.1 mM EDTA). After filling up to 5.0 ml of cell suspension with the same extraction buffer in 50 ml disposable centrifuge tube (IWAKI Glass, Tokyo, Japan), 1.5 ml of isogen-LS (Nippon gene, Toyama, Japan) and 10 grams of glass beads were added. Centrifuge tubes which contained the cell suspension with isogen-LS and glass beads was shaken with a horizontal table top shaker for an hour. In this step, 300 μg of total RNA was recovered.

[0076] Then, mRNA was purified by using an mRNA separation kit (Clontech). On 8.0 μg of mRNA from P. rhodozyma ATCC96594 and ATCC96815 strains were obtained.

[0077] To synthesize cDNA, we used the SMART cDNA construction kit (Clontech) according to the method specified by the manufacturer. We applied 2 μg of purified mRNA for a first strand synthesis followed by PCR amplification and obtained 1 mg of cDNA.

Example 8

Subcloning of pR16 and functional analysis of its insert fragment

[0078] The restriction map of pR16 is depicted in FIG. 2. Each EcoRI fragment whose length was 0.7 and 2.7 kb, was subeloned into pUC-G418 and named pRS913 and pRLR913, respectively.

[0079] Then, the astaxanthin-producing P. rhodozyma ATCC96594 strain was transformed with pRS913. As a result of this transformation study, yellow transformants were yielded. This suggested that 0.7 kb EcoRI fragment might contain a truncated AST gene and the transformation via a single-cross recombination between a 0.7 kb EcoRI fragment and its homologous sequence on the choromosome of P. rhodozyma would result in a gene disruption of the AST gene on the choromosome of P. rhodozyma.

[0080] Next, the beta-carotene-producing ATCC96815 strain was transformed with pRLR913 and red transformants were yielded. This suggested that the mutation point of strain ATCC96815 which led the astaxanthin-producing wild type strain to produce beta-carotene would lie in the 2.7 kb EcoRI fragment originally adjacent to the 0.7 kb EcoRI fragment in pR16.

[0081] Two hundred μg of cDNA prepared in Example 7 was subjected to agarose gel electrophoresis for virtual Northern analysis. In the case of the cDNAs prepared from ATCC96594 and 96815, two hands which, namely at 3.2 and 2.0 kb were hybridized in both cases by using the 2.7 kb EcoRI fragment of pRLR913 as a hybridization probe. This suggested that the ast mutation of ATCC96815 would be a point mutation which did not reflect to the change of the length of mRNA such as missense mutation.

[0082] In the case of using the 0.7 kb EcoRI fragment of pRS913 as a hybridization probe, a band of 2.0 kb was hybridized. From this study, it seemed that the AST gene might give a 2.0 kb transcript in P. rhodozyma.

Example 9

Cloning of the cDNA of the AST gene

[0083] To clone the cDNA for the AST gene from P. rhodozyma, we constructed cDNA library from P. rhodozyma ATCC96594. Total RNA was isolated by phenol extraction by combination of the cell disruption with glass beads as described in Example 7.

[0084] At first, cells of the ATCC96594 strain from 50 ml of a two-day-culture in YPD medium were harvested by centrifugation (1500 x g for 10 min.) and washed once with extraction buffer (100 mM Tris / HCl (pH 7.5) containing 0.1 M LiCl and 0.1 mM EDTA). After filling up to 5.0 ml of cell suspension with the same extraction buffer in 50 ml disposable centrifuge tube (IWAKI Glass), 1.5 ml of isogen-LS (Nippon gene) and 10 grams of glass beads were added. Centrifuge tubes which contained cell suspension with isogen-LS and glass beads were shaken with a horizontal table top shaker for an hour. In this step, 1.8 mg of total RNA was recovered.

[0085] Then, mRNA was purified by using the PolyATtract mRNA isolation system (Promega corp., Madison, USA) according to the method specified by the manufacturer. Finally, we obtained 8.0 μg of mRNA from the P. rhodozyma ATCC96594 strain.

[0086] To construct a cDNA library, 8.0 μg of the purified mRNA was used in the COPY kit (Invitrogen, Carlsbad, USA) with the protocol specified by the manufacturer. After ligation of a EcoRI adaptor (Stratagene), synthesized cDNA was subjected to agarose gel electrophoresis. After the excision of the agarose gel piece which covered the length of cDNAs from 1.9 to 2.3 kb, the collected cDNA species were purified by QIAEX II (QIAGEN). This size-fractionated cDNA was ligated to EcoRI-digested and dephosphorylated lambdaZAPII (Stratagene). The over-night ligation mixture was in vitro packaged with Gigapack III gold extract (Stratagene) and used for infection to E. coli XL1-Blue MRF' strain.

[0087] Conventional plaque screening was performed against 6000 plaques using 2.7 and 0.7 kb EcoRI fragment as described in Example 8 as hybridization probes. One plaque hybridized strongly to these probes, picked up with a sterilized toothpick and the eluted phage particle were used for in vivo excision, according to the method specified by the manufacturer. Finally, infected transformants of E. coli SOLR cells which showed resistance against ampicillin were isolated. After sequencing of the isolated plasmids obtained from these transformants, it turned out that these plasmids contained the same fragment as a part of the sequence of pR16 which was described in Example 6.

[0088] The entire sequence of the cDNA of the AST gene was determined and is shown as SEQ ID NO: 2 and its deduced amino acid sequence as SEQ ID NO: 1.

Example 10

Expression of AST gene in E. coli

[0089] To confirm that ORF for AST gene actually encode protein, expression study of AST gene was performed in E. coli expression system. At first, 6 x histidine (His) tag was added to carboxyl terminal end of AST product in order to make easy for following purification step. PCR primers whose sequences are listed in TABLE 1 were synthesized.

TABLE 1

PCR primers for cloning 3' portion of AST gene to which 6 x His tag is added

ast13; GTTCAAAGTTCATTTATGGA (sense primer) (SEQ ID NO: 4)
ast 14; GGATCCTCAGTGGTGGTGGTGGTGGTGTTCGACCGGCTTGACCTGCA (antisense primer) (SEQ ID NO: 5)

[0090] Next, 1.5 kb of NdeIEcoRI fragment of pAST1207 and 0.3 kb of EcoRIBamHI fragment of pAST114 were ligated into pET11c which was digested by NdeI and BamHI and ligated DNA was transformed into E. coli JM109 strain. Six independent ampicillin resistant clones were examined by restriction analysis and it was found that 5 of 6 clones had correct structure of recombinant expression plasmid containing AST gene. One of which was selected for further study (pAST120) and then transformed into E. coli BL21 (DE3) (pLysS) strain. It was revealed that all of the ampicillin resistant clones which were examined by restriction analysis possessed pAST120 properly. Next, expression study was performed by addition of 1mM IPTG to E.coli BL21 (DE3) (pLysS) (pAST120) growing culture when optical density (OD) at 600 nm reached to 0.8. After continuation of cultivation at 37 °C for 4 hours, cells were harvested by centrifugation and lysed by boiling in SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20 % glycerol, 4 % SDS, 0.005% bromophenol blue, 5 % mercaptoethanol). The lysate was then subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Expressed protein was not observed after staining by coomassie brilliant blue (Rapid stain CBB kit, nacalai tesque, Kyoto, JAPAN) (data not shown).

[0091] In general, it is reported that some modifications of amino acid sequence at amino terminal region of P450 protein is required to express P450 protein in E. coli expression system. In fact, AST gene which had an intact sequence was not expressed in E. coli (data not shown) and it was found that some modifications of sequence at amino terminal end was necessary in the case of AST gene as well as other P450 enzymes. As a next strategy for expression of recombinant AST gene, the construction, in which 6 x His tag sequence was added at amino terminal end of the AST protein on the deletion of the hydrophobic anchor sequences which were located at amino terminal end of AST gene, was made.

[0092] In order to add 6 x His tag sequence at amino terminal end of the AST protein on anchor sequences deleted at amino terminal end, following PCR primers were synthesized and used for PCR cloning.

TABLE 2

PCR primers for cloning AST gene lacking anchor sequence at its 5' portion to which 6 x His tag is added
ast32; CATATGCACCACCACCACCACCACCTGTATAACCTTCAGGGGCCC (sense primer for cloning of 5' end of AST gene) (SEQ ID NO: 6)
ast2; GTAACAACACCATCTCCGGT (antisense primer for cloning of 5' end of AST gene) (SEQ ID NO: 7)
ast13; GTTCAAAGTTCATTTATGGA (sense primer for cloning of 3' end of AST gene) (SEQ ID NO: 4)
ast33; GGATCCTCAACTCATTCGACCGGCTT (antisense primer for cloning of 3' end of AST gene) (SEQ ID NO: 8)

[0093] PCR condition was as follows; 25 cycles of 15 seconds at 94 °C, 30 seconds at 55 °C and 30 seconds at 72 °C. The plasmid, pAST1207 was used as PCR template. PCR fragments which had desired length were cloned into pCR2.1-TOPO (Invitrogen) and 6 independent clones which had expected inserts were examined for their insert sequence. As a result, two of the clones had exact insert sequence, respectively and one clone was selected and used for further study (pAST228 for 3' end of AST gene and pAST302#3202 for 5' end of AST gene, respectively). 0.2kb Nde-ISphI fragment from pAST302#3202, 1.5kb SphIEcoRI fragment from pAST1207 and 0.05kb KpnIBamHI fragment from pAST228 were ligated into pET11c digested by NdeI and BamHI and ligated mixture was transformed into E. coli DH5alpha. As a result of restriction analysis for 6 independent clones, it was found that all the clones had correct structure harboring AST gene for its expression. One clone was selected and used for further study (pAST315). Next, pAST315 was transferred into expression host E. coli BL21 (DE3) (pLysS). It was confirmed that all the 6 transformants had pAST315 correctly as a result of restriction analysis.

[0094] Next, expression study was performed by addition of 1.5 mM IPTG to E.coli BL21 (DE3) (pLysS) (pAST315) growing culture when optical density (OD) at 600 nm reached to 0.93. After continuation of cultivation at 37 °C for 4 hours, cells were harvested by centrifugation and lysed by boiling in SDS sample buffer. The lysate was then subjected to PAGE. Expressed protein whose molecular weight corresponded well with its deduced amino acid sequence (approximate 60 kDa) was observed after staining by coomassie brilliant blue (FIG. 3). From this result, it was confirmed that AST gene encode protein expected from its deduced open reading frame.

Example 11

In vitro characterization of the AST gene product

[0095] For the enzymatic characterization of the AST gene product, a standard assay which is used for the characterization of P450 enzymes can be applied. For this purpose, it is necessary that the reaction mixture contains a reconstituted membrane. As a reconstituted membrane, natural isolates such as mitochondrial membranes or microsomes and artificial membranes are often used. In any cases, it is necessary that an electron transfer between an electron acceptor and a receptor can occur. As an electron donor, cytochrome P450 reductase is often added to the reaction mixture. As an electron acceptor, oxygen molecules are involved. Under the presence of an electron source, such as reduced NADPH+, beta-carotene, which is a substrate of astaxanthin synthase can be converted to astaxanthin. Produced astaxanthin can be assayed qualitatively and quantitatively with HPLC analysis.

Example 12

Cloning of genomic fragment containing AST gene

[0096] To determine the genomic sequence containing AST gene, sequencing experiment performed by using primer-walking procedure. Sequencing analysis of pRS913 showed that pRS913 did not contain the 3' end of AST gene. To obtain the 3'-adjacent genomic fragment to AST gene, genome-walking experiment was performed. To do this, universal genome walker kit (Clontech) was exploited according to the method specified by the manufacturer. As a template of PCR, chromosomal DNA prepared in Example 1 was used. Gene specific primer, ast15 whose sequence was as listed in TABLE 3 was synthesized and used for PCR primer;

TABLE 3

Sequence of primer used for genome walking of AST gene
ast15; TAGAGAGAAGGAGGGGTACCAGATGC (SEQ ID NO.9)

[0097] PCR fragments which had appropriate length (smaller than 1 kb) were obtained from EcoRV and StuI library, purified and cloned into pCR2.1-TOPO (Invitrogen). As a result of sequencing, it was found that both fragments contained the genomic fragment contained AST gene. Based on the sequence which was located at 200 bp from polyA site for AST gene, PCR primer was designed as listed in TABLE 4.

TABLE 4

Sequence of primer for cloning of 3'-adjacent fragment to AST gene
ast18; CCCCGGATTGTGGAGAAACT (SEQ ID NO: 10)

[0098] By using ast15 and ast18 primers as PCR primers and chromosomal DNA prepared in Example 1 as PCR template, PCR was conducted. Proof-reading polymerase (HF polymerase, Clontech) ensured the amplification of PCR fragment which had exact sequence. PCR condition was as follows; 25 cycles of 15 seconds at 94 °C, 30 seconds at 55 °C and 30 seconds at 72 °C. Six independent clones which had 400 bp inserts showed the identical sequence.

[0099] By combined with sequences for pRS913 and pRL913, 3.9 kb sequence containing AST gene containing 474 bp of promoter region and 269 bp of terminator region was determined (SEQ ID NO. 3). As a result, AST gene showed intron-rich structure (17 introns).

Example 13

Determination of mutation point in beta-carotene producing strain, P. rhodozyma ATCC96815

[0100] To confirm the fact that beta-carotene production by P. rhodozyma ATCC96815 strain was caused from the mutation within the AST gene, genomic sequence containing AST gene obtained from ATCC96815 and its parent strain P. rhodozyma ATCC24230 were determined. To do this, PCR primers whose sequences were as listed in TABLE 5 were synthesized and used for PCR cloning;

TABLE 5

PCR primers for cloning entire genomic AST gene;
ast21; ATGTTCATCTTGGTCTTGCT (sense primer) (SEQ ID NO: 11)
ast4; ACGTAGAAGTCATAGCGCCT (antisense primer) (SEQ ID NO: 12)

[0101] By using HF polymerase (Clontech) as PCR polymerase and chromosomal DNA prepared from strains, ATCC96815 and ATCC24230 by the same protocol as Example 1 as PCR template, PCR was performed under the condition as follows; 25 cycles of 15 seconds at 94 °C, 30 seconds at 55 °C and 4 minutes at 72 °C. PCR fragments obtained whose length were approximate 3.5 kb were cloned into pCR2.1-TOPO and sequenced for their entire sequences by primer walking procedure. Between the sequence for P. rhodozyma ATCC96594 strain and ATCC24230 strain, 7 base changes were found. Four base changes were found in its exon sequence but those did not give any amino acid changes. Three base changes were found in its intron structure. In comparison between beta-carotene producing strain ATCC96815 and its parent strain, ATCC24230, one base change which was located at 5'-splicing sequence (GTAAGT > GTAAAT) within the eighth intron was found. This might indicate that mutation which conferred the phenotype of beta-carotene accumulation on astaxanthin-producing P. rhodozyma was caused from improper splicing of mRNA for AST gene.

[0102] To confirm this assumption, RT-PCR was performed by using cDNA prepared from P. rhodozyma ATCC96815 as PCR template. mRNA was isolated from P. rhodozyma ATCC96815 by the same protocol as Example 9 and used for the synthesis of cDNA. To obtain cDNA from this mRNA prepared from ATCC96815 by PCR method,

SMART PCR cDNA library construction kit (Clontech) was exploited according to the method specified by supplier. The following primers whose sequence were as listed in TABLE 6 and which covered eighth intron were synthesized and used for PCR primers.

TABLE 6

PCR primers for RT-PCR to detect the improper splicing product for AST gene
ast7; TTTGACTCAAGGATTAGCAG (sense primer) (SEQ ID NO: 13)
ast26; TGTCTTCTGAGAGTCGGTGA (antisense primer) (SEQ ID NO: 14)

[0103] RT-PCR condition was as follows; 25 cycles of 15 seconds at 94 °C, 30 seconds at 55 °C and 30 seconds at 72 °C. As a result of PCR, 300 bp of PCR products were amplified and cloned into pCR2.1-TOPO. Two independent clones which had 300 bp insert were sequenced. As a result, it was confirmed that improper splicing products for AST gene was synthesized in the P. rhodozyma ATCC96815 strain. Improper splicing for the eighth intron of AST gene might cause the production of shorter truncated AST protein than AST protein spliced properly because stop codon lay in the eighth intron. This result indicated that mutation point lay in AST gene which failed in the proper splicing.

Example 14

Expression of AST gene in beta-carotene-producing Phaffia rhodozyma

[0104] To confirm the fact that AST gene encoded the enzyme which was involved in the conversion from beta-carotene to astaxanthin, AST gene was cloned into beta-carotene-producing strain. To exclude the possibility of recombination at native locus of AST gene on the chromosome, expression plasmid for AST gene on AMY locus of Phaffia rhodozyma's chromosome was constructed. To do this, cloning of some genetic elements from Phaffia rhodozyma was required.

") Cloning of constitutive promoter and terminator from Phaffia rhodozyma

[0105] To clone a constitutive promoter and terminator from Phaffia rhodozyma, degenerate PCR method was exploited. Among the genes which are often used as constitutive promoter and terminator in yeast's genetics, TPI gene which encode triose phosphate isomerase was tried to be cloned. Among the conserved amino acid sequence registered in Blocks database (http://www.blocks.fhcrc.org/), two motif sequences (Arg-Thr-Phe-Phe-Val-Gly-Gly-Asn and Asp-Val-Asp-Gly-Phe-Leu-Val-Gly-Gly-Ala) were selected and their degenerate primers were synthesized as follows.

TABLE 7

Degenerate PCR primers for cloning of TPI gene from P. rhodozyma
tp1; MGNACNTTYTTYGTNGGNGGNAAY (sense primer) (SEQ ID NO: 15)
tp6; GCNCCNCCNACNARRAANCCRTCNACRTC (antisense primer) (SEQ ID NO: 16)
(M=A or C; N=A, C, G or T; Y=C or T; R=A or G)

[0106] PCR condition was as follows; 25 cycles of 15 seconds at 94 °C, 30 seconds at 46 °C and 15 seconds at 72 °C. ExTaq polymerase (Takara Shuzo) was used as PCR polymerase. As a PCR template, cDNA pool was prepared from mRNA isolated from P. rhodozyma ATCC96594 by using SMART PCR cDNA library construction kit (Clontech). 0.7 kb PCR fragment was purified and cloned into pCR2.1-TOPO. Six independent clones had inserts having desired length, judging from restriction analysis. Two of which were sequenced and it was confirmed that both of them had insert sequence which had striking homology to known TPI genes from various organisms. One of which was selected for further study (pTPI923).

[0107] Next, based on the insert sequence of pTPI923, several PCR primers whose sequences are listed in TABLE 8 were synthesized for genome walking to clone promoter and terminator of TPI gene. For this experiment, universal genome walker kit (Clontech) was exploited according to the method specified by the manufacturer.

TABLE 8

PCR primers for genome walking to clone TPI promoter and terminator
tp9; GCTTACCTCGCTTCCAACGTTTCCCAG (terminator cloning, primary) (SEQ ID NO: 17)
tp10; GGATCTGTCTCTGCCTCCAACTGCAAG (terminator cloning, nested) (SEQ ID NO: 18)
tp11; GGGTCAATGTCGGCAGCGAGAAGCCCA (promoter cloning, primary) (SEQ ID NO: 19)
tp12; ATGTACTCGGTAGCACTGATCAAGTAG (promoter cloning, nested) (SEQ ID NO: 20)

[0108] PCR condition was as follows; 7 cycles of 4 seconds at 94 °C and 3 minutes at 74 °C, followed by 32 cycles of 4 seconds at 94 °C and 3 minutes at 69 °C and succeeded to extension at 69 °C for 4 minutes. KOD polymerase (TOYOBO) was used as PCR polymerase. Chromosomal DNA prepared from P. rhodozyma ATCC96594 was used as a PCR template. As a result, candidate for terminator region was obtained from EcoRV and StuI library. Sequencing analysis for these candidates revealed that both clones had the downstream sequence for TPI gene containing deduced 3' end of the TPI structure gene and terminator region. In case of cloning for promoter region, candidates which were obtained from EcoRV library contained deduced 5' end of the TPI structure gene and promoter region.

[0109] Then, PCR primers whose sequences are listed in TABLE 9 were synthesized for the construction of promoter cassette and terminator cassette derived from TPI gene.

TABLE 9

PCR primers to construct TPI promoter and TPI terminator cassette
tp13; GCGGCCGCATCCGTCTCGCCATCAGTCT (sense primer for promoter cassette) (SEQ ID NO: 21)
tp14; CCTGCAGGTCTAGAGATGAATAAATATAAAGAGT (antisense primer for promoter cassette) (SEQ ID NO: 22)
tp15; CCTGCAGGTAAATATATCCAGGGATTAACCCCTA (sense primer for terminator cassette) (SEQ ID NO: 23)
tp16; GGTACCCGTGCGCAGTCGACCGAGACAT (antisense primer for terminator cassette) (SEQ ID NO: 24)

[0110] PCR condition was as follows; 25 cycles of 15 seconds at 94 °C, 30 seconds at 55 °C and 30 seconds at 72 °C. HF polymerase (Clontech) was used as PCR polymerase and yielded PCR fragments were cloned into pCR2.1-TOPO. As a result of restriction and sequencing analysis, it was found that clones which had identical sequences were obtained. Each one clone was selected for further study (pTPIP1104 for promoter cassette and pTPIT1104 for terminator cassette, respectively).

2) Cloning of partial amylase gene from Phaffia rhodozyma

[0111] To locate and express a foreign gene on the chromosome of P. rhodozyma, amylase gene was cloned from P. rhodozyma. In case that expression vector on which foreign gene would be cloned could contain homologous genetic fragment to the chromosomal sequence of P. rhodozyma such as amylase gene, expression vector can be integrated on the homologous region on chromosome of P. rhodozyma after the single cross recombination.

[0112] Eleven amino acid sequences for amylase from various organisms were selected from Entrez database (http://www.ncbi.nlm.nih.gov/Entrez/) and used for amino acid alignment by clustal W (Thompson, J.D., Higgins, D.G. and Gibson, T.J., Nucleic Acids Research, 22: 4673-4680, 1994). The eleven organisms whose amylase sequences were registered on the database are as listed in TABLE 10;

TABLE 10

Various amylase genes which were registered on database for clustal W analysis
Aspergillus niger var. awamori amyA gene (accession number X52755)
Aspergillus niger var. awamori amyB gene (accession number X52756)
Aspergillus kawachii acid-stable alpha-amylase gene (accession number AB008370)

TABLE 10 (continued)

Various amylase genes which were registered on database for clustal W analysis

Aspergillus oryzae amyl gene (accession number X12725)
Aspergillus shirousamii alpha-amylase gene (accession number P30292)
Cryptococcus species alpha-amylase gene (accession number D83541)
Lipomyces kononenkoae subsp. spencermartinsiae alpha-amylase gene (accession number U30376)
Debaryomyces occidentalis amy1 gene (accession number X16040)
Saccharomycopsis fibuligera ALP1 gene (accession number X05791)
Schizosaccharomyces pombe alpha-amylase gene (accession number Z64354)

[0113] Two conserved amino acid sequences (Asp-Tyr-Ile-Gln-Gly-Met-Gly-Phe-Asp/Thr-Ala-Ile-Trp and Asp-Gly-Ile-Pro-Ile-Ile-Tyr-Tyr-Gly-Thr-Glu-Gln) for amylase were selected to clone amylase gene from P. rhodozyma by degenerate PCR method. Then, PCR primers whose sequences are listed in TABLE 11 were synthesized for the cloning of AMY gene from P. rhodozyma.

TABLE 11

Degenerate PCR primers for cloning of amylase (AMY) gene from P. rhodozyma

amy1; GAYTAYATHCARGGNATGGGNTTYRMNGCNATHTG (sense primer) (SEQ ID NO: 25)
amy2; TGYTCNGTNCCRTARTADATDATNGGDATNCCRTC (antisense primer) (SEQ ID NO: 26)
(Y=C or T; H=A, C or T; R=A or G; N=A, C, G or T; M=A or C; D=A, G or T)

[0114] PCR condition was as follows; 25 cycles of 15 seconds at 94 °C, 30 seconds at 50 °C and 2 minutes at 72 °C. ExTaq polymerase (Takara Shuzo) was used as PCR polymerase. As a PCR template, chromosomal DNA prepared in Example 1 and cDNA pool prepared from mRNA isolated from P. rhodozyma ATCC96594 by using SMART PCR cDNA library construction kit (Clontech) were used. 1.7 kb and 0.9 kb PCR fragments were yielded when chromosome and cDNA were used as PCR template, respectively. Both fragments were purified and cloned into pCR2.1-TOPO. Six independent clones had inserts having desired length, judging from restriction analysis. Two of which were sequenced and it was confirmed that both of them had insert sequence which had striking homology to known amylase genes from various organisms. One of which contained chromosomal AMY fragment was selected for further study (pAMY216). To construct a partial amylase cassette, two PCR primers whose sequences are listed in TABLE 12 were synthesized based on the internal sequence of insert fragment of pAMY216.

TABLE 12

PCR primers to construct a partial AMY cassette

amyl101; CCGCGGCATTGATACCTCTACCCCGT (sense primer for AMY cassette) (SEQ ID NO:27)
amy102; GCGGCCGCCTGCAATCCTGGATCCACCG (antisense primer for AMY cassette) (SEQ ID NO: 28)

[0115] PCR condition was as follows; 25 cycles of 15 seconds at 94 °C, 30 seconds at 55 °C and 2 minutes at 72 °C. HF polymerase (Clontech) and chromosomal DNA were used as PCR polymerase and PCR template, respectively. The yielded PCR fragment was cloned into pCR2.1-TOPO. As a result of restriction and sequencing analysis, it was found that the clone which had correct sequence was obtained. One clone was selected for further study (pAMY1113).

3) Construction of expression vector for AST gene which functioned in Phaffia rhodozyma

[0116] Expression plasmid for AST gene was constructed by restriction digestion and ligation of each genetic component. At first, 0.3 kb KpnIPstI fragment from pTPIT1104 and 1.7 kb SacIKpnI fragment from pG418Sa512 were ligated into pGEM-T plasmid which was digested by SacI and PstI. It was found that 9 clones among 12 transformants had correct structure as a result of restriction digestion and one of those was selected for further study (pTPITG1120).

[0117] Next, PCR cloning for AST gene was performed to add the appropriate restriction site to both ends. PCR primers whose sequences are listed in TABLE 13 were synthesized.

TABLE 13

PCR primers to clone entire AST gene cassette
ast11; TCTAGAATGTTCATCTTGGTCTTGCTCA (sense primer) (SEQ ID NO: 29)
ast12; CCTGCAGGTCATTCGACCGGCTTGACCT (antisense primer) (SEQ ID NO: 30)

[0118] PCR condition was as follows; 25 cycles of 15 seconds at 94 °C, 30 seconds at 55 °C and 2 minutes at 72 °C. HF polymerase (Clontech) and pAST1207 were used as PCR polymerase and PCR template, respectively. The yielded PCR fragment was cloned into pCR2.1-TOPO. As a result of restriction and sequencing analysis, it was found that one clone which had correct sequence was obtained. This clone was selected for further study (pAST113).

[0119] Finally, 1.6 kb SacIINotI fragment from pAMY1113, 0.3 kb NotIXbaI fragment from pTPIP1104 and 1.5 kb XbaISse8387I fragment from pAST113 were ligated into pTPITG1120 which was digested by SacII and Sse8387I. It was confirmed that all the five transformants tested had correct structure as a result of restriction analysis and one of which was selected for further study (pAATG123).

4) Restoration of astaxanthin production in beta-carotene-producing Phaffia rhodozyma

[0120] The expression plasmid for AST gene (pAATG123) was transformed into beta-carotene-producing Phaffia rhodozyma ATCC96815. Biolistic transformation was performed as described in Example 3. Two geneticin-resistant colonies which colored red were picked up and selected for further study. In order to confirm the integration at AMY locus on the chromosome of P. rhodozyma, PCR primer whose sequence is listed in TABLE 14 was synthesized.

TABLE 14

PCR primer to confirm the integration of expression plasmid at AMY locus on the chromosome of P. rhodozyma
amy5; CTCTCCTGTTCACAAAAACA (sense primer) (SEQ ID NO: 31)

[0121] Chromosome was prepared from those transformants and used for PCR template. PCR condition was as follows; 25 cycles of 15 seconds at 94 °C, 30 seconds at 55 °C and 2 minutes at 72 °C. ExTaq polymerase (Takara Shuzo) was used as PCR polymerase. Positive 2.0 kb PCR band was yielded in the PCR reaction in which chromosome obtained from red-colored transformants was used as a template DNA. No PCR band was observed in the PCR reaction mixture in which chromosome derived from host strain, P. rhodozyma ATCC96815 was used as a PCR template.

5) Flask fermentation by recombinants in which recombinant AST gene was integrated on the chromosome of beta-carotene-producing P. rhodozyma

[0122] The productivity of astaxanthin was evaluated in the flask fermentation. The medium formulation for flask fermentation is as follows.

TABLE 15

Seed medium formulation for flask fermentation	
Glucose	30.0 g / l
NH_4Cl	4.83 g / l
KH_2PO_4	1.0 g/ l
$MgSO_4$-$7H_2O$	0.88 g / l

TABLE 15 (continued)

Seed medium formulation for flask fermentation	
NaCl	0.06 g / l
$CaCl_2$-$2H_2O$	0.2 g / l
KH phthalate	20.0 g / l
$FeSO_4$-$7H_2O$	28 mg / l
Citric acid-$1H_2O$	15.0 mg / l
$ZnSO_4$-$7H_2O$	40.0 mg / l
$CuSO_4$-$5H_2O$	0.75 mg / l
MnSO4-4,$5H_2O$	0.6 mg / l
H_3BO_3	0.6 mg / l
Na_2MoO_4-$2H_2O$	0.6 mg / l
KI	0.15 mg / l
Myo-inositol	60.0 mg / l
Nicotinic acid	3.0 mg / l
Ca D-pantothenate	3.0 mg / l
Vitamin B1 (thiamin HCl)	3.0 mg / l
p-Aminobenzoic acid	1.8 mg / l
Vitamin B6 (pyridoxine HCl)	0.3 mg / l
Biotin	0.048 mg / l
7 ml / Test Tube (21 mm diameter)	

TABLE 16

Medium formulation for flask fermentation	
$MgSO_4$-$7H_2O$	2.1 g / l
$CaCl_2$-$2H_2O$	0.865 g / l
$(NH_4)_2SO_4$	3.7 g / l
$FeSO_4$-$7H_2O$	0.28 g / l
Glucose (sterilized separately)	22 g / l
KH_2PO_4 (sterilized separately)	14.25 g / l
Citric acid-$1H_2O$	0.21 g / l
$ZnSO_4$-$7H_2O$	70.14 mg / l
$CuSO_4$-$5H_2O$	10.5 mg / l
$MnSO_4$-4,$5H_2O$	8.4 mg / l
H_3BO_3	8.4 mg / l
Na_2MoO_4-$2H_2O$	8.4 mg / l
KI	2.1 mg / l
Myo-inositol	0.374 g / l

TABLE 16 (continued)

Medium formulation for flask fermentation	
Nicotinic acid	18.7 mg / l
Ca D-pantothenate	28.05 mg / l
Vitamin B1 (thiamin HCl)	18.7 mg / l
p-Aminobenzoic acid	11.22 mg / l
Vitamin B6 (pyridoxine HCl)	1.87 mg / l
Biotin	1.122 mg /
CaCO3	10 g / l

1 drop of Actcol (Takeda Chemical Industries Ltd., Osaka, JAPAN) was added to each flask.

50ml (final volume with 5% of seed inoculum) / 500 ml flask with buffles

[0123] Cells were harvested from fermented broth after 7-day fermentation and analyzed for their accumulation of astaxanthin and beta-carotene by HPLC as described in Example 4. Results are summarized in TABLE 17.

TABLE 17

Restoration of astaxanthin production by the recombinants in which AST gene was integrated. (Data is indicated as relative titer of astaxanthin and beta-carotene against the titer of beta-carotene accumulated by P. rhodozyma ATCC96815)

Strain	Relative titer (%)	
	Astaxanthin	Beta-carotene
ATCC96815 :: pR16	34.0 %	18.6 %
ATCC96815 :: pAATG123	16.3 %	56.3 %
ATCC96815	0 %	100 %

[0124] Partial restoration of astaxanthin production by ATCC96815 :: pAATG123 indicated that promoter strength by TPI promoter is not strong enough for perfect restoration of astaxanthin production.

SEQUENCE LISTING

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<220>
<223> Description of Artificial Sequence: sense primer
for expresion of AST gene in E. coli

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<210> 5
<211> 47
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: antisense primer for expression of AST gene in E. coli

<400> 5
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<210> 6
<211> 45
<212> DNA
<213> Artificial Sequence

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<223> Description of Artificial Sequence: 5' sense primer for expression of modified AST gene in E. coli

<400> 6
catatgcacc accaccacca ccacctgtat aaccttcagg ggccc 45

<210> 7
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: 5' antisense primer for expression of modified AST gene in E. coli

<400> 7
gtaacaacac catctccggt 20

<210> 8
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: 3' antisense primer for expression of modified AST gene in E. coli

<400> 8
ggatcctcaa ctcattcgac cggctt 26

<210> 9
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: genome walking primer for cloning of AST gene

<400> 9
tagagagaag gaggggtacc agatgc 26

<210> 10
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: antisense primer for cloning of terminator region of AST gene

<400> 10
ccccggattg tggagaaact 20

<210> 11
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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: sense primer for cloning genomic AST gene

<400> 11
atgttcatct tggtcttgct 20

<210> 12
<211> 20
<212> DNA
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<220>
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<400> 12
acgtagaagt catagcgcct 20

<210> 13
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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: sense primer for RT-PCR of AST gene

<400> 13
tttgactcaa ggattagcag 20

<210> 14
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: antisense primer for RT-PCR of AST gene

<400> 14
tgtcttctga gagtcggtga 20

<210> 15
<211> 24
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<220>
<223> Description of Artificial Sequence: degenerate sense primer for cloning of TPI gene

<400> 15
mgnacnttyt tygtnggngg naay 24

<210> 16
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<212> DNA
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<220>
<223> Description of Artificial Sequence: degenerate antisense primer for cloning of TPI gene

<400> 16
gcnccnccna cnarraancc rtcnacrtc 29

<210> 17
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primary walking primer for cloning of TPI terminator

<400> 17
gcttacctcg cttccaacgt ttcccag 27

<210> 18
<211> 27
<212> DNA
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<220>
<223> Description of Artificial Sequence: nested walking primer for cloning of TPI terminator

<400> 18
ggatctgtct ctgcctccaa ctgcaag 27

<210> 19
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primary walking primer for cloning og TPI promoter

<400> 19
gggtcaatgt cggcagcgag aagccca 27

<210> 20
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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: nested walking primer for cloning of TPI promoter

<400> 20
atgtactcgg tagcactgat caagtag 27

<210> 21
<211> 28
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<220>
<223> Description of Artificial Sequence: sense primer for construction of TPI promoer cassette

<400> 21
gcggccgcat ccgtctcgcc atcagtct 28

<210> 22
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<220>
<223> Description of Artificial Sequence: antisense primer for construction of TPI promoter cassette

<400> 22
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<210> 23
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: sense primer for construction of TPI terminator cassette

<400> 23
cctgcaggta aatatatcca gggattaacc ccta 34

<210> 24
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: antisense primer for construction of TPI terminator cassette

<400> 24
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<210> 25
<211> 35
<212> DNA
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<220>
<223> Description of Artificial Sequence: degenerate sense primer for cloning of AMY gene

<400> 25
gaytayathc arggnatggg nttyrmngcn athtg 35

<210> 26
<211> 35
<212> DNA
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<220>
<223> Description of Artificial Sequence: degenerate antisense primer for cloning of AMY gene

<400> 26
tgytcngtnc crtartadat datnggdatn ccrtc 35

<210> 27
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: sense primer for construction of a partial AMY cassette

<400> 27
ccgcggcatt gatacctcta ccccgt 26

<210> 28
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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: antisense primer for construction of a partial AMY cassette

<400> 28
gcggccgcct gcaatcctgg atccaccg 28

<210> 29
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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: sense primer for construction of AST cassette

<400> 29
tctagaatgt tcatcttggt cttgctca 28

<210> 30
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<220>
<223> Description of Artificial Sequence: antisense primer for construction of AST cassette

<400> 30
cctgcaggtc attcgaccgg cttgacct 28

<210> 31
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: sense primer for confirmation of integration at AMY locus by PCR analysis

<400> 31
ctctcctgtt cacaaaaaca 20

Claims

1. An isolated DNA comprising a nucleotide sequence coding for an enzyme having astaxanthin synthase activity catalyzing the reaction from beta-carotene to astaxanthin, preferably in P. rhodozyma.

2. The isolated DNA according to claim 1, which is characterized in that

   (a) the said nucleotide sequence codes for said enzyme having the amino acid sequence shown in SEQ ID NO: 1, or
   (b) the said nucleotide sequence codes for a variant of the said enzyme selected from (i) an allelic variant or (ii) an enzyme having one or more amino acid addition, insertion, deletion and/or substitution but having still the same type of enzymatic activity.

3. The isolated DNA according to claim 1, which is characterized in that the said nucleotide sequence is:

   (i) a nucleotide sequence represented in SEQ ID NO: 2;
   (ii) a nucleotide sequence which, because of the degeneracy of the genetic code, encodes an astaxanthin synthase having the same amino acid sequence as that encoded by the nucleotide sequence (i); and
   (iii) a nucleotide sequence which hybridizes to the complement of the nucleotide sequence from i) or ii) under standard hybridizing conditions.

4. The isolated DNA according to claim 1, which is characterized in that the said nucleotide sequence is:

   (i) a nucleotide sequence represented in SEQ ID NO: 3;
   (ii) a nucleotide sequence which, because of the degeneracy of the genetic code, encodes an astaxanthin synthase having the same amino acid sequence as that encoded by the nucleotide sequence (i); and
   (iii) a nucleotide sequence which hybridizes to the complement of the nucleotide sequence from i) or ii) under standard hybridizing conditions.

5. A vector or plasmid comprising the isolated DNA as claimed in anyone of claims 1 to 4.

6. A host cell transformed or transfected by the isolated DNA as claimed in any one of claims 1 to 4 or a vector or plasmid as claimed in claim 5.

7. A polypeptide encoded by the isolated DNA as claimed is anyone of claims 1 to 4.

8. A process for the production of a polypeptide having an activity of astaxanthin synthase which comprises culturing a transformed host cell as claimed in claim 5 under the conditions conductive to the production of said enzyme.

9. A process for the biological production of astaxanthin which comprises introducing one or more isolated DNA as claimed in any one of claims 1 to 4 into an appropriate host organism, cultivating the obtained organism under the condition conductive to the production of astaxanthin and recovering astaxanthin from the culture.

10. A process for the production of astaxanthin which comprises contacting beta-carotene with the polypeptide having an activity of astaxanthin synthase in the presence of an appropriate electron donor in an appropriate reaction mixture containing an appropriate reconstituted membrane.

11. The process according to claim 10, wherein the polypeptide is present in the form of a reconstituted membrane which is prepared from a biological membrane like a microsome or a mitochondrial membrane.

12. The process according to claim 10, wherein the polypeptide is present in the form of a reconstituted artificial membrane, like a liposome.

13. The process according to claim 10, wherein the said electron donor is an appropriate electron donor which can reduce its reaction center, like the cytochrome P450 reductase.

FIG.1 Biosynthetic pathway from GGPP to astaxanthin in the bacterial sytem which produces astaxanthin

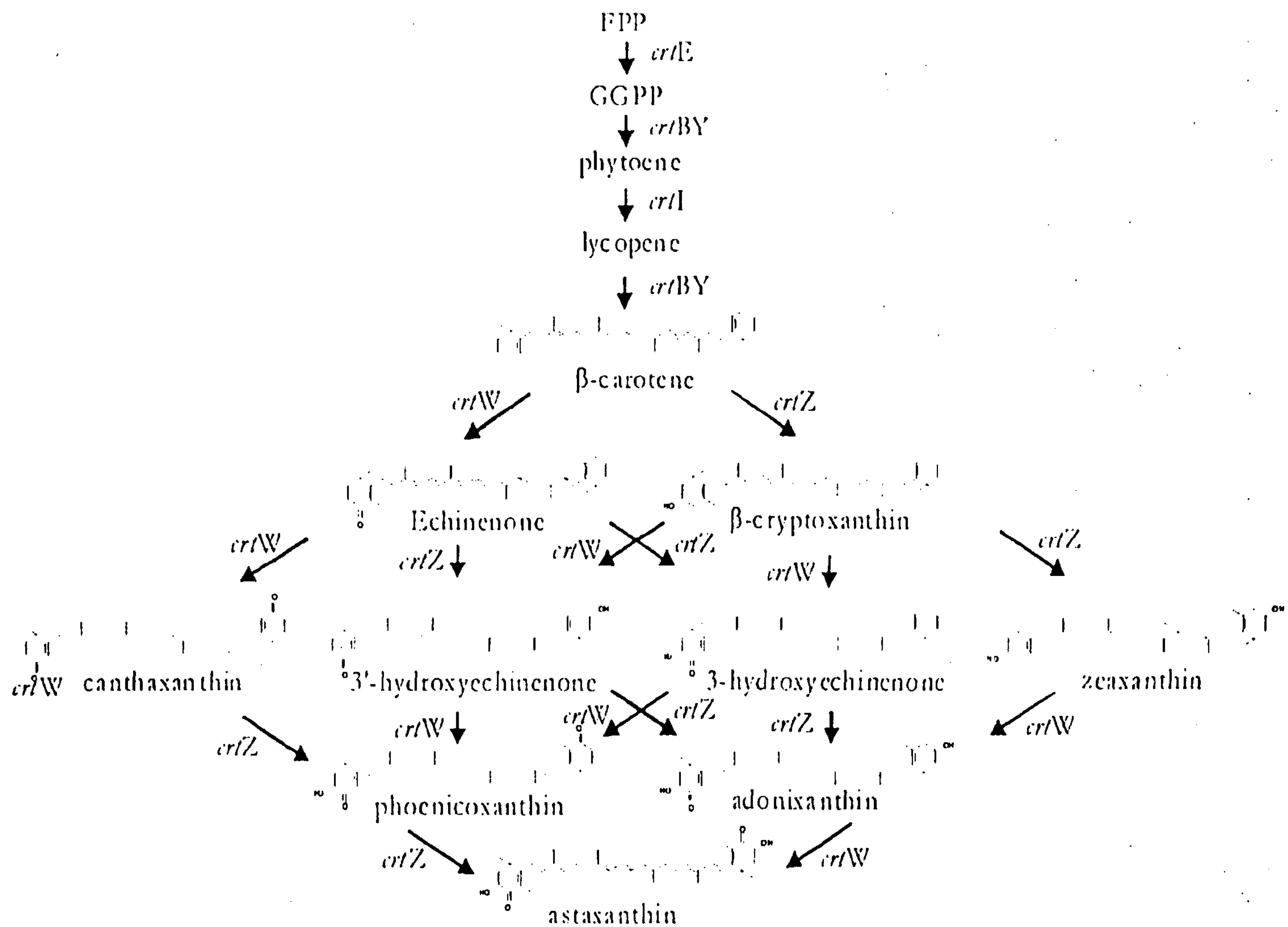
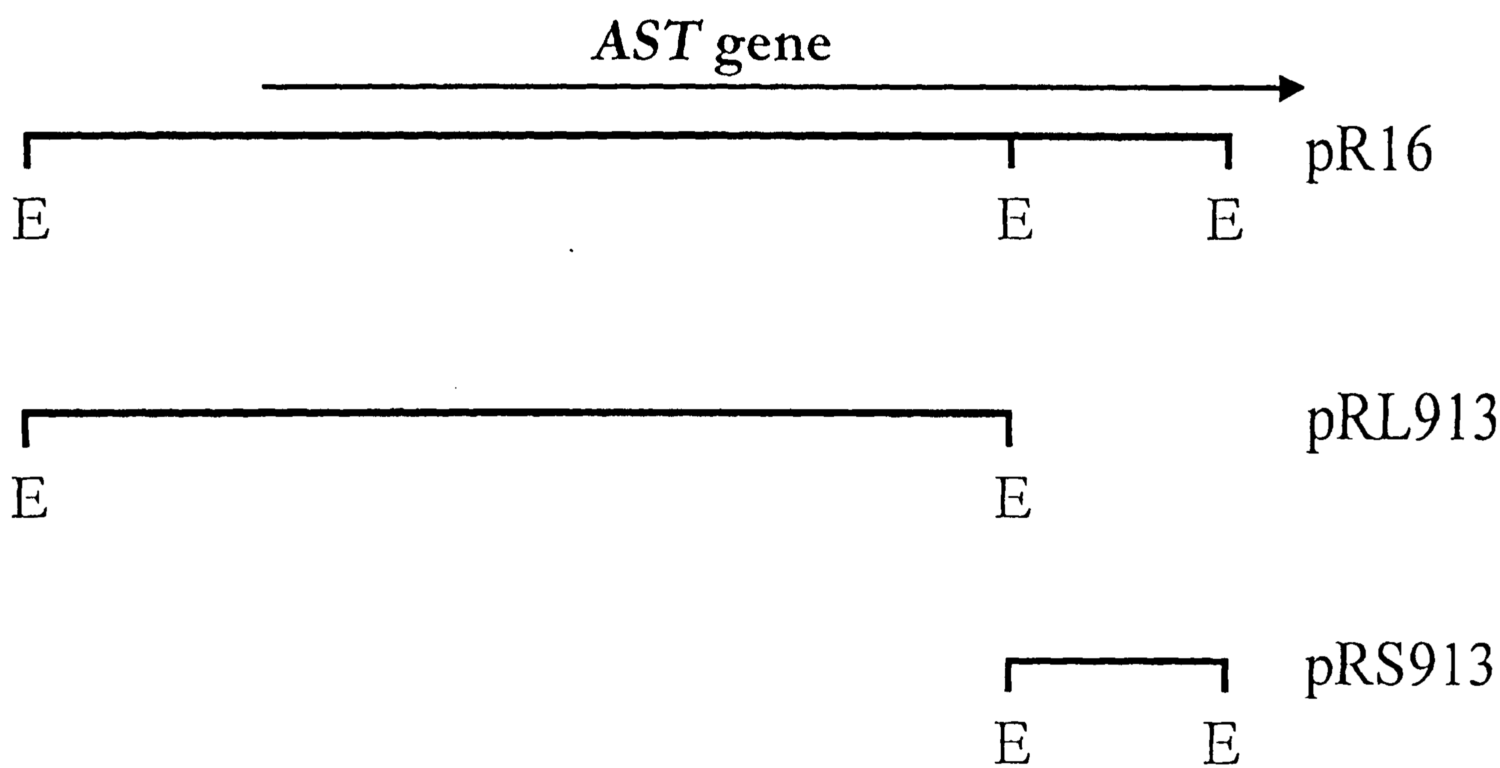


FIG. 2 Restriction map of DNA fragments which were used for functional analysis of *AST* gene from *Phaffia rhodozyma*

Vector backbone of these plasmids was pUC-G418. Restriction enzyme indicated as E is *Eco*RI.



European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 00 10 4430

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
D,A	WO 97 23633 A (GIST BROCADES BV ;OOIJEN ALBERT JOHANNES JOSEPH (NL); VERDOES JAN) 3 July 1997 (1997-07-03) * page 9, line 6 - page 10, line 20 * * page 25; examples 9-19 * * claims *	1-13	C12N15/52 C12N9/00 C12P23/00
D,A	EP 0 769 551 A (KIRIN BREWERY) 23 April 1997 (1997-04-23) * examples 1,2,4 *	1-13	
D,A	WERY J ET AL: "HIGH COPY NUMBER INTEGRATION INTO THE RIBOSOMAL DNA OF THE YEAST PHAFFIA RHODOZYMA" GENE, vol. 184, no. 1, 1997, pages 89-97, XP000677654 ISSN: 0378-1119		

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

C12P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	10 July 2000	Andres, S

CATEGORY OF CITED DOCUMENTS

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E : earlier patent document, but published on, or after the filing date
D : document cited in the application
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The members are as contained in the European Patent Office EDP file on
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10-07-2000

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			CA	2241267	A	03-07-1997
			EP	0870042	A	14-10-1998
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			AU	685354	B	15-01-1998
			AU	4889996	A	02-10-1996
			NO	964754	A	08-01-1997
			WO	9628545	A	19-09-1996

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82